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The Role of Short Homology Repeats and TdT in Generation of the Invariant γδ Antigen Receptor Repertoire in the Fetal Thymus

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Summary

Fetal thymic and adult epithelial Vγ3$^+$ and Vγ4$^+$ T cells express γδ antigen receptors (TCR) with invariant junctions lacking N nucleotides. Using transgenic recombination substrates, we show that di- or trinucleotide repeats, either in the coding region or in P elements, have strong effects on the site of recombination. In other mice bearing a terminal deoxynucleotidyl transferase (TdT) transgene under the control of the CD2 promoter, we found that the frequency of canonical junctions was markedly reduced with a concomitant increase in in-frame noncanonical junctions with N nucleotides. Together, our results show that short homology repeats direct the site of rearrangement and thus play a critical role in the generation of γδ T cell receptor canonical junctions. Increased TdT activity in Vγ3$^+$ T cells has a inhibitory effect on junctional homogeneity in these cells.

Introduction

Diversity in immunoglobulins and T cell antigen receptors (TCR) is generated through V(D)J rearrangement. The use of different germline gene segments, the imprecise joining of gene segments, the addition of nongermline-encoded nucleotides, and the combinatorial association of the protein products of the rearranged genes allows potential diversity that has been estimated as approaching 10^{11} for immunoglobulins, 10^{16} for αβ TCR, and 10^{18} for γδ TCR (Davis and Bjorkman, 1988). For αβ T cells, it is clear that the functional repertoire in an individual is shaped by a complex process of negative and positive selection to eliminate self-reactive clones and ensure the survival of clones with the capacity to recognize peptide antigens in the context of self-major histocompatibility antigens (Robey and Fowlkes, 1994). The nature of the forces that shape the γδ TCR repertoire is much less clear, particularly for those which reside in the epithelial tissues.

In the epithelia of the skin and reproductive tract of mice there are unique populations of γδ T cells with very restricted TCR repertoire (Asarnow et al., 1988, 1989; Itohara et al., 1990; Nandi and Allison, 1991). In the skin, essentially all the T cells express γ chains encoded by Vγ3–Jγ1 segments, while those of the reproductive tract are encoded by Vγ4–Jγ1 segments. In both cases, the junctions are characterized by minimal diversity, and the absence of nongermline-encoded nucleotides. In these tissues there is a single and identical predominant δ chain encoded by the Vδ1–Dδ2–Jδ2 segments. In all mouse strains examined to date, greater than 90% of the productively rearranged γ and δ genes in these tissues have the same canonical junctions. These observations raise important questions concerning the function and origin of these essentially monomorphic TCR. It has been suggested that these epithelial T cells perform a unique form of immunological surveillance for damage-induced self-antigens (Allison and Havran, 1991; Raulet et al., 1991). The concept of trauma signal surveillance has gained support with the demonstration that skin-derived Vγ3$^+$ T cells can recognize heat-stressed keratinocytes (Havran et al., 1991).

The precursors of epithelial Vγ3$^+$ and Vγ4$^+$ T cells are produced in a series of overlapping waves during fetal thymic development (Havran and Allison, 1988; Allison and Havran, 1991). Moreover, Vγ3$^+$ cells are produced only during fetal development in a process that require both fetal stem cells and fetal thymic microenvironment (Havran and Allison, 1990; Ikuta et al., 1990). The potential TCR repertoires of these fetal precursors is constrained by the restricted expression and ordered rearrangement of V genes during development (Goldman et al., 1993), as well as by the absence of the diversifying effect of the addition of random nucleotides at the junction by terminal deoxynucleotidyl transferase (TdT) (Landau et al., 1987; Rothenberg and Triglia, 1983). Despite these constraints, there still exists a considerable potential for diversification either by recombination at different sites or by exonucleolytic processing of the coding ends.

It has been suggested that the canonical repertoire is generated by the effects of cellular selection (Itohara and Tonegawa, 1990). However, two recent reports suggested that cellular selection may not be required for the formation of the invariant γδ repertoires in Vγ3$^+$ and Vγ4$^+$ T cells. Using a rearrangement-competent transgene with a frameshift mutation in the Vγ3 segment upstream of the joints, it was demonstrated that the transgenic Vγ3–Jγ1 canonical junctions are still formed even though the transgene can not generate functional Vγ3 protein (Asarnow et al., 1993). Similarly, it was found that the Vγ3/Jγ1 and Vγ4/Jγ1 canonical junctions were generated in normal frequency in TCR δ gene mutant mice (Itohara et al., 1993). These results give support to the previous suggestion that the invariant repertoire is a result of directed rearrangement, rather than cellular selection (Raulet et al., 1991; Allison and Havran, 1991). However, the exact mecha-

nisms involved in the generation of these canonical junctions remain to be determined.

Previous results have shown a correlation between the presence of short homology repeats near the ends of the coding segments and the appearance of predominant junctional sequences in immunoglobulin H (IgH) gene rearrangement (Gu et al., 1990a, 1990b). Short homology repeats were suggested to be responsible for the limited junctional diversity of neonatal immunoglobulin gene rearrangement (Feeney, 1991a, 1992). It has been proposed that these direct repeats may help to align the broken ends during the recombination process (Alt and Baltimore, 1982; Roth and Wilson, 1986; Lieber, 1992). AT/ATA short repeats are observed at the Vγ3–Jγ1, Vγ4–Jγ1, and Vδ1–Dδ2 junctions in TCR γ and δ chain rearrangement (Allison and Havran, 1991; Raulet et al., 1991). Similarly, AG/GAG/GGA short repeats generated by P element addition appear to be present at the Dδ2–Jδ2 and Dδ2–Jδ1 junctions (Itohara et al., 1993). Since the junctional diversity at all these joints is severely restricted, it seemed possible that short homology repeats might promote the formation of certain types of joints and thus contribute to the generation of canonical junctions. However, there has been no direct evidence linking the presence of short homology repeats and the formation of γδ canonical junctions.

The level of TdT activity during early fetal thymic development may also influence the generation of canonical junctions. TdT is thought to be the major enzyme activity responsible for N region addition (Alt and Baltimore, 1982). Mutations in murine TdT gene resulted in total loss of N nucleotides in the V–D–J recombination junctions of both B and T lymphocytes (Komori et al., 1993; Gilfillan et al., 1993). Loss of TdT enzymatic activity is also considered to be responsible for the higher frequency of in-frame canonical and out-of-frame predominant Vγ3–Jγ1 junctions in the thymus of adult mice (Komori et al., 1993; Gilfillan et al., 1993). A low level of TdT enzyme activity in fetal and neonatal spleen and liver B cells has also been suggested to be responsible for the lack of N region diversity in immunoglobulin recombination junctions (Opstelten et al., 1986; Meek, 1990; Feeney, 1990, 1991b, 1993). It was recently shown that presence of TdT activity could diminish the effect of homology-directed rearrangement in an in vitro recombination system (Gerstein and Lieber, 1993). In fetal thymic development, although TdT expression is weakly detectable in bulk fetal thymocyte populations as early as day 15 of ontogeny, its expression is absent in the fetal Vγ3$^+$ thymocytes (J. Noble and J. P. A., unpublished data). These data suggest that lack of TdT enzyme activity in certain populations of fetal thymocytes may further restrict the repertoire diversity and contribute to the formation of canonical Vγ3–Jγ1 and Vγ4–Jγ1 rearrangements.

Here, we examine the role of an AT direct repeat present near the ends of the Vγ3 and Jγ1 coding segments on the generation of Vγ3/Jγ1 canonical junctions using a transgenic recombination substrate containing framshift mutations in the Vγ3 and Vγ4 coding segments (Asarnow et al., 1993). Point mutations were introduced into this rearrangement substrate near the 3′ end of Vγ3 coding segment to destroy the AT short repeat or introduce new types of short repeats. Our results indicate that AT/ATA short repeats are sufficient to direct the Vγ3/Jγ1 rearrangement and formation of the Vγ3/Jγ1 canonical junctions. The influence of TdT enzyme activity on the formation of the Vγ3 invariant repertoire was assessed by generating mice carrying a transgene that expresses TdT under the CD2 promoter. The junction diversity and the frequency of N nucleotide addition in Vγ3/Jγ1 junctions were significantly increased in TdT transgenic mice. These data provide direct evidence that short repeats can direct the site of recombination in Vγ3/Jγ1 rearrangement, and that TdT expression can disrupt the process. Thus, the canonical Vγ3 junctions appear to be the result of rearrangement directed by short repeats in the absence of TdT.

Results

Role of AT Short Repeats in Directing the Vγ3 Rearrangement

To determine the function of AT short repeat near the ends of the Vγ3/Jγ1 coding segments in generating the γδ canonical sequences, we used a rearrangement substrate that contains the Vγ4, Vγ3–Jγ1–Cγ1 coding segments in the germline configuration (Figure 1A). Termination codons have been introduced into the Vγ4 and Vγ3 coding segments to prevent the production of Vγ4 and Vγ3 protein products. Previous results showed that the Vγ3/Jγ1 canonical junctions were generated at the same frequency in the transgenic substrate rearrangement as in the endogenous Vγ3 gene rearrangement (Asarnow et al., 1993). Point mutations were introduced near the 3′ end of Vγ3 coding segment in the recombination substrate to destroy specifically the AT direct repeat or to generate new direct repeats (Figure 1B). Recombination substrates carrying the various mutations were injected into fertilized eggs to generate transgenic mice. Day 17 transgenic fetal thymic DNA was polymerase chain reaction (PCR) amplified using primers designed to amplify specifically either transgenic or endogenous V–J rearrangement junctions.

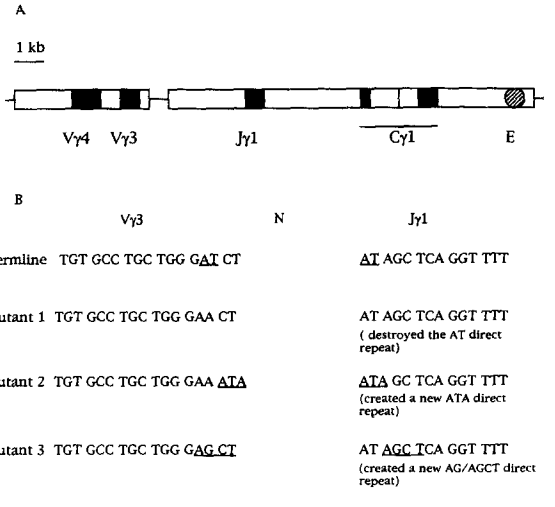


Figure 1. TCR γ Gene Rearrangement Substrates

(A) Schematic diagram of the TCR γ gene rearrangement substrate.
(B) Point mutations generated near the 3′ end of Vγ3 coding segment.

Destruction of the AT Short Repeat Eliminates the Generation of Vγ3/Jγ1 Canonical Junction

In mutant 1, the AT dinucleotide near the 3′ end of the Vγ3 gene segment was changed to AA, thus destroying the direct repeat with the AT at the 5′ end of the Jγ1 segment (Figure 1B). The sequences of Vγ3/Jγ1 as well as Vγ4/Jγ1 junctions of the rearranged transgene in the day 17 fetal thymi are shown in Figure 2. Recombination at the site corresponding to the wild-type canonical junction was not observed among any of the 29 individual Vγ3 junctional sequences examined in mice carrying mutant 1 substrate. This suggests a critical role of the AT direct repeat in generating the Vγ3 canonical junction (Figure 2A). The canonical Vγ4–Jγ1 sequence was generated in all 8 in-frame Vγ4 rearrangements of the 15 individual Vγ4 rearrangement junctions examined, indicating that mutation at the Vγ3 junction had no effect on the neighboring Vγ4 gene rearrangement in the recombination substrate (Figure 2B).

One effect of the destruction of the AT repeats was a marked reduction in the frequency of rearrangements in which the V and J segments were juxtaposed in the appropriate translational reading frame. While approximately one-third of rearrangements were in frame in the endogenous genes of nontransgenic littermates (See Figure 3C) or in the unmutated recombination substrate (Asarnow et al., 1993), mutant 1 yielded only 2 of 29 in-frame Vγ3/Jγ1 rearrrangements.

Although the elimination of the AT direct repeat prevented formation of the canonical junction, several types of out-of-frame junctions were repeatedly obtained (Figure 2A). For instance, the junctional sequence indicated as type III repeated five times. It is notable that this sequence could be directed by new TA or TAG repeats generated by addition of the P nucleotides A or AG at the 3′ end of the Vγ3 segment (Figure 2A). Similarly, the junction indicated as type IV could also be attributed to a TA repeat generated by P nucleotide addition at the ends of the Vγ3 and Jγ1 segments. Short repeats formed by the P nucleotide addition have been previously suggested by several laboratories to direct the VDJ rearrangement in both fetal thymocytes and neonatal B cells (Raulet et al., 1991; Asarnow et al., 1993, Itohara et al., 1993, Komori et al., 1993, Chukwuocha et al., 1995). Our results suggest that even in the absence of an AT direct repeat in the coding segments, short repeats formed by the P element addition can still direct the rearrangement, although the resulting predominant rearrangements are mostly out of frame. These results support a role for the short repeats in directing the site of the V–J rearrangement.

Other types of junctional sequences were also generated at relatively high frequency (Figure 2A, types V, VI). The mechanisms involved in the generation of these types of junctions are not clear, since they could not be generated through directed rearrangement by either germline direct repeats or repeats generated by P nucleotide addition at the precise ends of the coding sequences. It is generally accepted that P nucleotides are generated by the asymmetrical opening of the hairpin structure, which seals the coding and signal ends immediately after the double-stranded DNA cleavage (Lafaille et al., 1989). However, if the hairpins could be formed not only at the exact junctional breakpoint but also at coding ends where one or two nucleotides are removed from the junctional breakpoint, then the asymmetrical opening of these hairpins could form a new type of P nucleotides, referred to as recessed P nucleotide. The AT short repeats formed by these recessed P nucleotides could explain the generation of these predominant junctional sequences (Figure 2A, types V, VI). More examples of the AT repeats directed rearrangements of this type could be found in the junctions of mutant 2 and 3 transgenic fetuses (Figure 3A, types IV, V, VI, VII; see Figure 4, type X).

Creation of a New ATA Repeat Generates a New Type of Predominant Vγ3/Jγ1 Junctional Sequence

To determine whether the AT short repeat is sufficient to direct the γδ gene rearrangement, we replaced the CT dinucleotide sequence at the end of the Vγ3 coding sequence of mutant 1 with the trinucleotide ATA. This substrate, mutant 2, thus has a 3 nt repeat with the 5′ end of

A

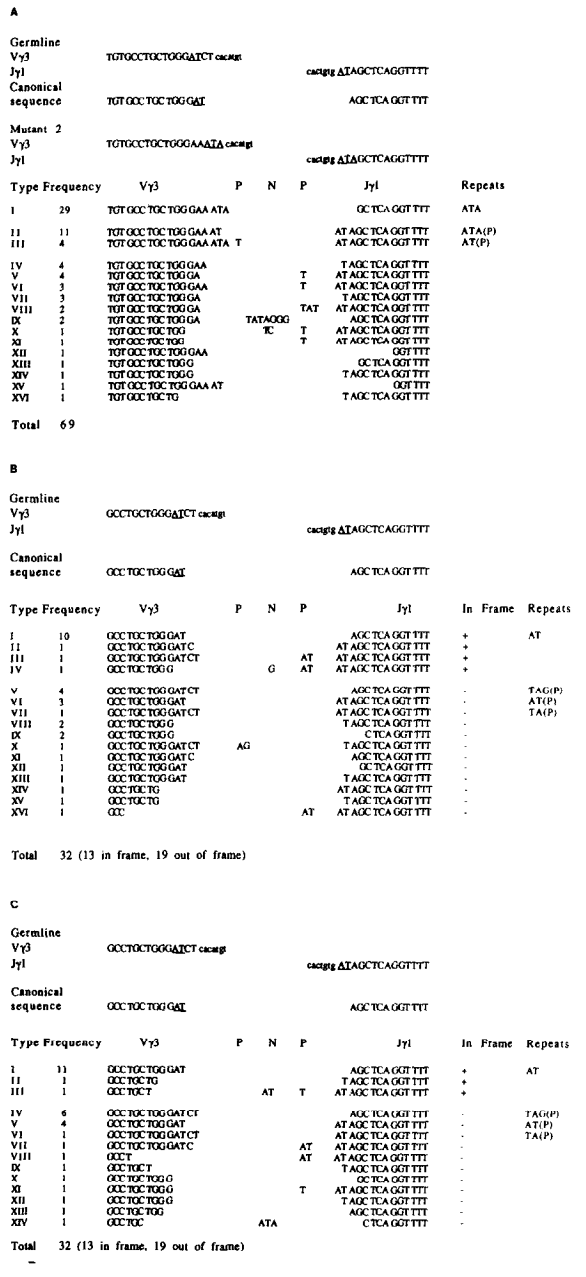
Germline Vγ3		GCCTGCTGGGATCT cacagt						
Jγ1						cactgtg ATAGCTCAGGTTTT		
Canonical sequence		GCC TGC TGG GAT				AGC TCA GGT TTT		
Mutant 1 Vγ3		GCCTGCTGGGAACT cacagt						
Jγ1						cacagtg ATAGCTCAGGTTTT		
Type	**Frequency**	**Vγ3**	**P**	**N**	**P**	**Jγ1**	**In Frame**	**Repeats**
I	1	GCC TGC TGG GAA CT	A			AGC TCA GGT TTT	+	
II	1	GCC TGC TGG G				GC TCA GGT TTT	+	
III	5	GCC TGC TGG GAA CT				AGC TCA GGT TTT	-	TAG(P)
IV	2	GCC TGC TGG GAA CT				AT AGC TCA GGT TTT	-	TA(P)
V	4	GCC TGC TGG GAA				T AGC TCA GGT TTT	-	
VI	3	GCC TGC TGG GA			T	AT AGC TCA GGT TTT	-	
VII	4	GCC TGC TGG G				T AGC TCA GGT TTT	-	
VIII	1	GCC TGC TGG GA			TAT	AT AGC TCA GGT TTT	-	
IX	1	GCC TGC TGG GAA CT	AG			T AGC TCA GGT TTT	-	
X	1	GCC TGC TGG GAA				AT AGC TCA GGT TTT	-	
XI	1	GCC TGC TGG GAA		C		AGC TCA GGT TTT	-	
XII	1	GCC TGC TGG GAA		TC		AT AGC TCA GGT TTT	-	
XIII	1	GCC TGC TGG GA		TC	AT	AT AGC TCA GGT TTT	-	
XIV	1	GCC TGC TGG G				AGC TCA GGT TTT	-	
XV	1	GCC TGC TGG				AGC TCA GGT TTT	-	
XVI	1	GCC TGC T			AT	AT AGC TCA GGT TTT	-	
Total	29 (2 in frame, 27 out of frame)							

B

Germline Vγ4		TGTGCATGCTGGGATA cacagtg						
Jγ1						cactgtg ATAGCTCAGGTTTT		
Canonical sequence		TGT GCA TGC TGG GATA				GC TCA GGT TTT		
Type	**Frequency**	**Vγ4**	**P**	**N**	**P**	**Jγ1**	**In Frame**	**Repeats**
I	8	TGT GCA TGC TGG GAT A				GC TCA GGT TTT	+	ATA
II	5	TGT GCA TGC TGG GAT A				T AGC TCA GGT TTT	-	AT(P)
III	1	TGT GCA TGC			AT	AT AGC TCA GGT TTT	-	
IV	1	TGT GCA TGC TGG GAT A	TA	GAGGGTGG		AGC TCA GGT TTT	-	
Total	15 (8 in frame, 7 out of frame)							

Figure 2. Nucleotide Sequences of the Transgenic Vγ3 Junctions in Mice Carrying Mutant 1 Rearrangement Substrate

Junctional sequences of the transgenic Vγ3–Jγ1 rearrangement (A) and Vγ4–Jγ1 rearrangement (B) in the day 17 fetal thymus of mice bearing mutant 1 transgenic substrate. The sequences are aligned with the germline sequences of TCR Vγ3 and Vγ4 segments (Garman et al., 1986). The recombination signal sequences are shown in lowercase letters. The AT direct repeats are underlined. The canonical sequence(s) represent the predominant Vγ3–Jγ1/Vγ4–Jγ1 junctional sequence(s) in both fetal thymus and adult epithelial tissues (Allison and Havran; 1991). The short repeats that could contribute to the generation of certain types of junctions are listed in a separate column titled Repeats. Repeats that may have formed after the P nucleotides addition are marked as (P). The junctional nucleotides were assigned following the general order of germline → P nucleotides → N nucleotides. Junctions are considered to be in-frame if the same open reading frame as the canonical Vγ3–Jγ1 junction is maintained.



A

Germline
Vγ3 TGTGCCTGCTGGGATCT cacagt
Jγ1 cactgg ATAGCTCAGGTTTT
Canonical sequence TGT GCC TGC TGG GAT AGC TCA GGT TTT

Mutant 2
Vγ3 TGTGCCTGCTGGGAAATA cacagt
Jγ1 cactgg ATAGCTCAGGTTTT

Type	Frequency	Vγ3	P	N	P	Jγ1	Repeats
I	29	TGT GCC TGC TGG GAA ATA				GC TCA GGT TTT	ATA
II	11	TGT GCC TGC TGG GAA AT				AT AGC TCA GGT TTT	ATA(P)
III	4	TGT GCC TGC TGG GAA ATA	T			AT AGC TCA GGT TTT	AT(P)
IV	4	TGT GCC TGC TGG GAA				T AGC TCA GGT TTT	
V	4	TGT GCC TGC TGG GA			T	AT AGC TCA GGT TTT	
VI	3	TGT GCC TGC TGG GAA			T	AT AGC TCA GGT TTT	
VII	3	TGT GCC TGC TGG GA				T AGC TCA GGT TTT	
VIII	2	TGT GCC TGC TGG GA			TAT	AT AGC TCA GGT TTT	
IX	2	TGT GCC TGC TGG GA		TATAGGG		AGC TCA GGT TTT	
X	1	TGT GCC TGC TGG		TC	T	AT AGC TCA GGT TTT	
XI	1	TGT GCC TGC TGG			T	AT AGC TCA GGT TTT	
XII	1	TGT GCC TGC TGG GAA				GGT TTT	
XIII	1	TGT GCC TGC TGG G				GC TCA GGT TTT	
XIV	1	TGT GCC TGC TGG G				T AGC TCA GGT TTT	
XV	1	TGT GCC TGC TGG GAA AT				GGT TTT	
XVI	1	TGT GCC TGC TG				T AGC TCA GGT TTT	

Total 69

B

Germline
Vγ3 GCCTGCTGGGATCT cacagt
Jγ1 cactgg ATAGCTCAGGTTTT

Canonical sequence GCC TGC TGG GAT AGC TCA GGT TTT

Type	Frequency	Vγ3	P	N	P	Jγ1	In Frame	Repeats
I	10	GCC TGC TGG GAT				AGC TCA GGT TTT	+	AT
II	1	GCC TGC TGG GAT C				AT AGC TCA GGT TTT	+	
III	1	GCC TGC TGG GAT CT			AT	AT AGC TCA GGT TTT	+	
IV	1	GCC TGC TGG G		G	AT	AT AGC TCA GGT TTT	+	
V	4	GCC TGC TGG GAT CT				AGC TCA GGT TTT	-	TAG(P)
VI	3	GCC TGC TGG GAT				AT AGC TCA GGT TTT	-	AT(P)
VII	1	GCC TGC TGG GAT CT				AT AGC TCA GGT TTT	-	TA(P)
VIII	2	GCC TGC TGG G				T AGC TCA GGT TTT	-	
IX	2	GCC TGC TGG G				C TCA GGT TTT	-	
X	1	GCC TGC TGG GAT CT	AG			T AGC TCA GGT TTT	-	
XI	1	GCC TGC TGG GAT C				AGC TCA GGT TTT	-	
XII	1	GCC TGC TGG GAT				GC TCA GGT TTT	-	
XIII	1	GCC TGC TGG GAT				T AGC TCA GGT TTT	-	
XIV	1	GCC TGC TG				AT AGC TCA GGT TTT	-	
XV	1	GCC TGC TG				T AGC TCA GGT TTT	-	
XVI	1	GCC			AT	AT AGC TCA GGT TTT	-	

Total 32 (13 in frame, 19 out of frame)

C

Germline
Vγ3 GCCTGCTGGGATCT cacagt
Jγ1 cactgg ATAGCTCAGGTTTT

Canonical sequence GCC TGC TGG GAT AGC TCA GGT TTT

Type	Frequency	Vγ3	P	N	P	Jγ1	In Frame	Repeats
I	11	GCC TGC TGG GAT				AGC TCA GGT TTT	+	AT
II	1	GCC TGC TG				T AGC TCA GGT TTT	+	
III	1	GCC TGC T		AT	T	AT AGC TCA GGT TTT	+	
IV	6	GCC TGC TGG GAT CT				AGC TCA GGT TTT	-	TAG(P)
V	4	GCC TGC TGG GAT				AT AGC TCA GGT TTT	-	AT(P)
VI	1	GCC TGC TGG GAT CT				AT AGC TCA GGT TTT	-	TA(P)
VII	1	GCC TGC TGG GAT C			AT	AT AGC TCA GGT TTT	-	
VIII	1	GCC T			AT	AT AGC TCA GGT TTT	-	
IX	1	GCC TGC T				T AGC TCA GGT TTT	-	
X	1	GCC TGC TGG G				GC TCA GGT TTT	-	
XI	1	GCC TGC TGG G			T	AT AGC TCA GGT TTT	-	
XII	1	GCC TGC TGG G				T AGC TCA GGT TTT	-	
XIII	1	GCC TGC TGG				AGC TCA GGT TTT	-	
XIV	1	GCC TGC		ATA		C TCA GGT TTT	-	

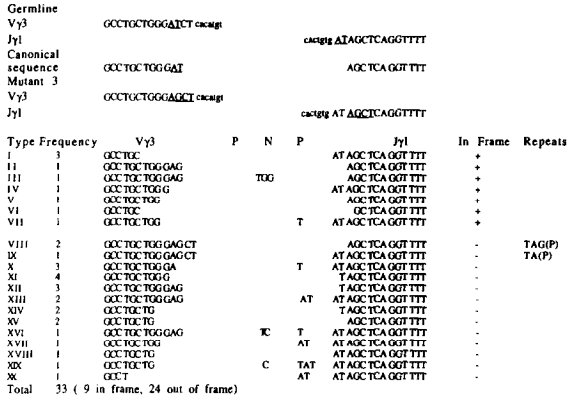
Total 32 (13 in frame, 19 out of frame)

Figure 3. Nucleotide Sequences of Vγ3 Junctions in Mice Carrying Mutant 2 Rearrangement Substrate

Junctional sequences of the transgenic (A) and the endogenous (B) Vγ3–Jγ1 rearrangements in the day 17 fetal thymus of mice bearing mutant 2 transgenic substrate. (C) Junctional sequences of the Vγ3–Jγ1 rearrangements in day 17 fetal thymus of the nontransgenic control mice.

the Jγ1 segment (see Figure 1B), a feature shared with the Vγ4 gene.

Mice carrying mutant 2 transgenic substrate generated a new type of Vγ3/Jγ1 predominant junction (Figure 3A, type I). Out of 69 total junctions examined, 29 contain this type of Vγ4-like junction. These data suggest that AT short repeats are sufficient to direct the site of the Vγ3/Jγ1 gene rearrangement. The endogenous Vγ3/Jγ1 junctions in mice carrying the mutant 2 transgene as well as their non-



Germline
Vγ3 GCCTGCTGGGATCT cacagt
Jγ1 cactgg ATAGCTCAGGTTTT
Canonical sequence GCC TGC TGG GAT AGC TCA GGT TTT
Mutant 3
Vγ3 GCCTGCTGGGAGCT cacagt
Jγ1 cactgg AT AGCTCAGGTTTT

Type	Frequency	Vγ3	P	N	P	Jγ1	In Frame	Repeats
I	3	GCC TGC				AT AGC TCA GGT TTT	+	
II	1	GCC TGC TGG GAG				AGC TCA GGT TTT	+	
III	1	GCC TGC TGG GAG		TGG		AGC TCA GGT TTT	+	
IV	1	GCC TGC TGG G				AT AGC TCA GGT TTT	+	
V	1	GCC TGC TGG				AGC TCA GGT TTT	+	
VI	1	GCC TGC				GC TCA GGT TTT	+	
VII	1	GCC TGC TGG			T	AT AGC TCA GGT TTT	+	
VIII	2	GCC TGC TGG GAG CT				AGC TCA GGT TTT	-	TAG(P)
IX	1	GCC TGC TGG GAG CT				AT AGC TCA GGT TTT	-	TA(P)
X	3	GCC TGC TGG GA			T	AT AGC TCA GGT TTT	-	
XI	4	GCC TGC TGG G				T AGC TCA GGT TTT	-	
XII	3	GCC TGC TGG GAG				T AGC TCA GGT TTT	-	
XIII	2	GCC TGC TGG GAG			AT	AT AGC TCA GGT TTT	-	
XIV	2	GCC TGC TG				T AGC TCA GGT TTT	-	
XV	2	GCC TGC TG				AGC TCA GGT TTT	-	
XVI	1	GCC TGC TGG GAG		TC	T	AT AGC TCA GGT TTT	-	
XVII	1	GCC TGC TGG			AT	AT AGC TCA GGT TTT	-	
XVIII	1	GCC TGC TG				AT AGC TCA GGT TTT	-	
XIX	1	GCC TGC TG		C	TAT	AT AGC TCA GGT TTT	-	
XX	1	GCC T			AT	AT AGC TCA GGT TTT	-	

Total 33 (9 in frame, 24 out of frame)

Figure 4. Nucleotide Sequences of the Transgenic Vγ3 in Mice Carrying Mutant 3 Rearrangement Substrate

Junctional sequences of the transgenic Vγ3–Jγ1 rearrangements in the day 17 fetal thymus of mice bearing mutant 3 transgenic substrate. The recombination signal sequences are shown in lowercase letters. The AGCT direct repeats are underlined.

transgenic littermates were also sequenced using primers that only amplify the endogenous Vγ3/Jγ1 rearrangement (Figures 3B and 3C). In transgenic fetuses carrying mutant 2 substrate, 10 out of 13 in-frame endogenous Vγ3/Jγ1 rearrangements contained the canonical junction, while in the nontransgenic fetus, 11 out of 13 in-frame endogenous Vγ3/Jγ1 rearrangements were of the canonical sequence (Figures 3B and 3C). The comparable frequency of the Vγ3 canonical junction in the rearrangements of endogenous genes in these mice suggests that the presence of the transgenic substrate had no general effect on the rearrangement machinery.

Two types of additional predominant junctional sequences were also generated in these mice, type II and III (Figure 3A). Again, the high frequency appearance of these two junctional sequences could be attributed to the ATA and AT repeats generated by P nucleotide addition.

AG or AGCT Repeats Failed to Direct the Vγ3/Jγ1 Rearrangement

From the data discussed above, it seems quite clear that AT or ATA repeats are sufficient for the generation of γδ canonical sequences. It was of interest to determine whether short repeats with other sequences could also direct rearrangement. The AT repeat near the 3′ end of the Vγ3 coding segment was changed to AG to form AG, AGC, or AGCT direct repeats with sequences in the Jγ1 coding segment (see Figure 1B). Mice carrying this mutated transgenic substrate, mutant 3, failed to produce any predominant transgenic rearrangement (Figure 4). The resultant transgenic junctions were quite diverse, although two types of out-of-frame junctions could be generated through directed rearrangement by potential TA(G)/TA repeats formed by P nucleotide addition (Figure 4, types VIII and IX). These results suggest that AG or AGCT repeats cannot direct the Vγ3 gene rearrangement efficiently. Similarly, a pair of AG direct repeats at the Vγ2/Jγ1 junction also fail to direct the Vγ2 gene rearrangement (Lafaille et al., 1989). However, we cannot rule out the possibility that the position of the AG repeat at the Vγ3/Jγ1 junction is

Table 1. Comparison of the Vγ3–Jγ1 Junctional Diversity and N Region Insertion in Control and TdT Transgenic Mice Thymus: Fetal Vγ3–Jγ1 Junctions in TdT Transgenic and Control Mice Thymus

Mice	Frameshift	Predominent junctions (percent)[a]	N nucleotide insertion (percent)	Junctional diversity[b]
Control mice[c]	In frame	38/48 (80%)	2/48 (4%)	8/48 (0.16)
	Out of frame	38/72 (53%)	3/72 (4%)	24/72 (0.33)
TdT transgenic mice	In frame	10/20 (50%)	6/20 (30%)	11/20 (0.55)
	Out of frame	15/29 (52%)	9/29 (31%)	16/29 (0.55)

[a] The predominant junctions in the in-frame rearrangements represent the canonical junctions. The predominant junctions in the Vγ3 out-of-frame rearrangements represent two types of junctions, which are frequently observed (Figure 3C, types IV and V).
[b] Junctional diversity is defined as the number of different functions in a group of rearrangement junctions divided by the total number of junctions in that group.
[c] Vγ3 rearrangements in the control mice were obtained from Figures 3B and 3C as well as from TdT nontransgenic littermates (Data unpublished).

also important for its function in directing rearrangement. The AG dinucleotide is located 3 nt away from the 5′ end of the Jγ1 coding sequence. Since in normal mice the sites of recombination are usually upstream of the AGCT sequence in Jγ1, it is possible that recombination at this site is generally precluded by the recombinase (see Figures 2–4; Figure 6; Asarnow et al., 1993, Itohara et al., 1993).

Formation of the Vγ3/Jγ1 Canonical Junction Is Impaired in TdT Transgenic Mice

Nongermline-encoded nucleotides are not present in canonical Vγ3 and Vγ4 junctions, and are rarely observed in any Vγ3 and Vγ4 junctions in the fetal thymus (Allison and Havran, 1991). As shown in Figure 3C, we found only two junctions containing nongermline nucleotides in a total of 32 Vγ3/Jγ1 rearrangements. Previous studies have reported the occurrence of N nucleotides in only about 6% of out-of-frame Vγ3 junctions in the fetal thymus, while in-frame fetal Vγ3 junctions contain essentially no N regions (Table 2). It is thought that the lack of N nucleotides may be due to a low level of TdT expression in the early fetal thymus. We therefore sought to determine whether expression of TdT in the early fetal thymus would affect the formation of canonical Vγ3/Jγ1 junctions. Transgenic mice were produced using a construct in which expression of TdT was regulated by the CD2 promoter (Figure 5A). This promoter was chosen because CD2 is expressed by fetal Vγ3$^+$ cells and can be detected on fetal thymocytes by day 15 of ontogeny (Duplay et al., 1989; Owen et al., 1988).

The activity of the CD2–HMTdT construct (Figure 5A) was tested in vitro by transient expression in transfected EL-4 cells, a mouse T cell lymphoma line. CD2–HMTdT-transfected T cells contain 5-fold higher TdT activity as compared with the untransfected T cells (data not shown). Cytohistology staining using a polyclonal anti-TdT antibody indicated that TdT enzyme in the transfected cells was localized mostly in the cytosol (data not shown).

Flow cytometric analysis of thymocytes from TdT transgenic and nontransgenic littermates showed that Vγ3$^+$ cells developed in normal numbers and that the level of Vγ3$^+$ TCR expression was unaffected in the TdT transgenic mice (data not shown)

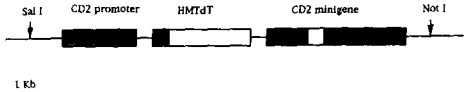
Analysis of the Vγ3 rearrangements in day 17 fetal thymus of the TdT nontransgenic littermates showed typical features of the Vγ3 rearrangements: dominant presence of the Vγ3 canonical junctions (13 out of 16) in the in-frame rearrangements and absence of the N nucleotides (data not shown). However, in the TdT transgenic fetal thymi, we observed a significant increase in the appearance of nongermline nucleotides in the junctions of both Vγ3 in-frame and out-of-frame rearrangements (Figure 5B). Approximately 30% of both in-frame and out-of-frame Vγ3 junctions contain N nucleotides in these thymi (Table 1). These N nucleotides contain a high percentage of G/C (65%), a general feature of the TdT involved N nucleotide addition.

Table 2. Comparison of the Vγ3–Jγ1 Junctional Diversity and N Region Insertion in Control and TdT Transgenic Mice Thymus: Reported Vγ3–Jγ1 Junctions in Fetal, Newborn, and Adult Mice Thymus

Mice	Frameshift	Predominant junctions (perent)[a]	N nucleotide Insertion (percent)	Junctional diversity[b]
Fetal Vγ3–Jγ1 junctions	In frame	49/54 (91%)	0/54 (0%)	4/54 (0.08)
	Out of frame	31/53 (59%)	3/53 (6%)	20/53 (0.36)
Newborn Vγ3–Jγ1 junctions	In frame	14/17 (82%)	0/17 (0%)	4/14 (0.28)
	Out of frame	15/35 (43%)	5/35 (14%)	18/35 (0.51)
Adult Vγ3–Jγ1 junctions	In frame	13/32 (41%)	15/32 (47%)	20/32 (0.63)
	Out of frame	13/55 (24%)	35/55 (63%)	43/55 (0.78)

[a] The predominant junctions in the in-frame rearrangements represent the canonical junctions. The predominant junctions in the Vγ3 out-of-frame rearrangements represent two types of junctions, which are frequently observed (Figure 3C, types IV and V).
[b] Junctional diversity is defined as the number of different junctions in a group of rearrangement junctions divided by the total number of junctions in that group.
The reported fetal Vγ3–Jγ1 junctions were obtained from Lafaille et al. (1990) and Itohara et al. (1993). Newborn Vγ3–Jγ1 junctions were obtained from Asarnow et al. (1993). Adult Vγ3–Jγ1 junctions were obtained from Komori et al. (1993).

A



B

Germline
Vγ3 GCCTGCTGGGATCT cacagtg
Jγ1 cactgtg ATAGCTCAGGTTTT
Canonical sequence GCCTGCTGGGAT AGCTCAGGTTTT

Type	Frequency	Vγ3	P	N	P	Jγ1	In Frame	Repeats
I	10	GCCTGCTGGGAT				AGCTCAGGTTTT	+	AT
II	1	GCCTGCTGGGATC				ATAGCTCAGGTTTT	+	
III	1	GCCTGCTGGGG		GG		AGCTCAGGTTTT	+	
IV	1	GCCTGCTGGGAT		CCCC		ATAGCTCAGGTTTT	+	
V	1	GCCTGCTGGGAT		GGAG		GCTCAGGTTTT	+	
VI	1	GCCTGCTGGGA		A		AGCTCAGGTTTT	+	
VII	1	GCCTGCTGGG		TT	T	ATAGCTCAGGTTTT	+	
VIII	1	GCCTGCTGGGATCT				TAGCTCAGGTTTT	+	
IX	1	GCCTGCTGG				TCAGGTTTT	+	
X	1	GCCTGCTGGGATCT			AT	ATAGCTCAGGTTTT	+	
XI	1	GCCTGCTGG		ATATTAGAG		AGCTCAGGTTTT	+	
XII	2	GCCTGCTGGGATCT				AGCTCAGGTTTT	-	TAG(P)
XIII	8	GCCTGCTGGGAT				ATAGCTCAGGTTTT	-	AT(P)
XIV	1	GCCTGCTGGGATCT			T	ATAGCTCAGGTTTT	-	
XV	1	GCCTGCTGGGA				ATAGCTCAGGTTTT	-	
XVI	1	GCCTGCTGGGAT				TAGCTCAGGTTTT	-	
XVII	1	GCCTGCTGGGAT		AG		ATAGCTCAGGTTTT	-	
XVIII	1	GCCTGC		GG	T	ATAGCTCAGGTTTT	-	
XIX	1	GCCTGCTGGGG		TCCG		ATAGCTCAGGTTTT	-	
XX	1	GCCTGCTGGGATC		GAG		AGCTCAGGTTTT	-	
XXI	1	GCCTGCTGGGG		CCTCC		TAGCTCAGGTTTT	-	
XXII	1	GCCTGCTGGG				AGCTCAGGTTTT	-	
XXIII	1	GCCTGCTGGGATC		ATG		AGCTCAGGTTTT	-	
XXIV	1	GCCTGCTGGGAT		CC		CAGGTTTT	-	
XXV	1	GCCTGCTGGGATC				AGCTCAGGTTTT	-	
XXVI	1	GCCTGCTGGGATCT	A	GGG		AGCTCAGGTTTT	-	
XXVII	1	GCCTGCTGGG		CCC	AT	ATAGCTCAGGTTTT	-	
Total	49 (20 in frame, 29 out of frame)							

Figure 5. The Effects of TdT on Vγ3–Jγ1 Junctional Diversity

(A) Schematic diagram of the transgenic construct that expresses the mouse–human hybrid terminal deoxynucleotidyl transferase (TdT).
(B) The junctional sequences of the Vγ3–Jγ1 rearrangement in day 17 fetal thymus of the TdT transgenic mice.

The frequency of canonical Vγ3-Jγ1 junctions was reduced from the expected 80%–95% observed in control mice to about 50% in the TdT transgenic mice, suggesting a partially impaired ability to generate the canonical junctions in these mice. The generation of noncanonical in-frame Vγ3 junctions could be attributed mostly to the presence of TdT activity, since 6 out of 10 of these junctions contained N nucleotides (Figure 5B).

Our results indicate that introduction of a TdT transgene into mice increases the junctional diversity of fetal Vγ3 rearrangement. Junctional diversity can be defined as the number of different junctions in a group of rearrangement junctions divided by the total number of junctions in that group. According to this definition, the fetal Vγ3 junctions in the TdT transgenic mice have a diversity of 0.55 in both in-frame and out-of-frame rearrangements (Table 1), much higher than that observed in control fetal Vγ3 rearrangements, which is around 0.08–0.16 for the in-frame rearrangements and 0.33–0.36 for the out-of-frame rearrangements (Table 1). The junctional diversity of Vγ3 rearrangements in fetal TdT transgenic mice is even higher than that in the newborn mice thymus but lower than that in the adult thymus (Tables 1 and 2). These results indicate that enhanced TdT expression can increase the frequency of N nucleotide addition as well as the junctional diversity at the Vγ3/Jγ1 junction. Thus, lack of TdT activity during gene rearrangement in the precursors of the $Vγ3^+$ and $Vγ4^+$ fetal thymocytes may contribute to the generation of γδ canonical junctions by restricting the repertoire diversity.

Discussion

We have examined two factors that may contribute to the generation of γδ TCR canonical repertoires: the presence of short sequence repeats near the ends of coding segments and TdT activity. The function of the AT short repeat near the ends of Vγ3 and Jγ1 coding segments was examined using mutated rearrangement substrates that contained the Vγ4, Vγ3, Jγ1, Cγ1 gene segments. Introduction of these substrates into mice allowed us to study the V–J rearrangement in the absence of TCR-mediated positive and negative cellular selections. The rearrangement of these substrates during fetal thymic development occurs independently but closely reflects the endogenous Vγ gene rearrangement events. Using this system, we found that AT or ATA direct repeats are sufficient to direct the Vγ3/Jγ1 gene rearrangement and to generate canonical junctions. Our results give further support to the idea that positive selection at the cellular level is not required for the generation of the canonical rearrangement.

There has been some controversy as to whether short homology repeats can direct the V–(D)–J rearrangement (Lewis, 1994, see discussion). Although several reports indicated that the presence of short direct repeats could lead to severely restricted junctional diversity, others failed to reach the same conclusion (Gerstein and Lieber, 1993; Chukwuocha et al., 1995; Boubnov et al., 1993). It was also reported that certain predominant neonatal immunoglobulin gene rearrangements are generated through developmental selection rather than homology-directed rearrangement (Pandey et al., 1993). However, it is clear that cellular selection is not required for the predominant generation of the canonical Vγ3–Jγ1 and Vγ4–Jγ1 junctions in fetal thymus, since these junctions are observed even when TCRs are not expressed on cell surface (Asarnow et al., 1993; Itohara et al., 1993). Our results provide direct evidence that AT/ATA repeats dictate the sites of the Vγ3–Jγ1 rearrangement. Introduction of a ATA repeat at a different position of the Vγ3 segment altered the site of Vγ3–Jγ1 rearrangement and generated a new type of predominant junction (Figure 3A). The position of the AT repeat may also safeguard the rearrangements to maintain proper reading frame, since the elimination of the AT repeat drastically reduced the frequency of in-frame Vγ3–Jγ1 rearrangements (Figure 2A).

Several types of predominant out-of-frame Vγ3/Jγ1 rearrangements have been frequently observed (Raulet et al., 1991; Asarnow et al., 1993, Lafaille et al., 1989; Komori et al., 1993). It was previously suggested that short repeats formed by P nucleotide addition might contribute to the formation of these predominant junctional sequences. Our results indicate that repeats generated by P nucleotide addition can still direct the rearrangement and form several types of out-of-frame predominant junctions even when the AT repeat in the germline coding region is not present (Figure 2A; Figure 4). These results give further support to the idea that short repeats are crucial elements in directing γ gene rearrangement.

It is not clear whether short repeats with other se-

quences can also direct rearrangement. In our system, AG or AGCT repeats could not effectively direct the Vγ3/Jγ1 rearrangement (Figure 4). Although AG-rich repeats have been suggested to direct the Dδ2/Jδ2 rearrangement (Itohara et al., 1993), AG direct repeats at the Vγ2/Jγ1 junction did not generate a high frequency of the corresponding rearrangement (Lafaille et al., 1989). It may be that the sequence content of the short homology is important in determining whether it is able to direct the rearrangement effectively. Another possibility, which we favor, is that the position of the short homology is crucial. Thus, if a short repeat is located too far from the ends of the coding segments, particularly the end of the Jγ1 segment, it may not be able to direct the rearrangement. In a particular V–(D)–J recombination system, both the sequence content and the position of a short repeat may determine whether it can effectively direct the rearrangement. This may also provide an explanation to the discrepancy in the literature regarding whether short homology repeats can direct the rearrangement (reviewed by Lewis, 1994).

It is not known how short homology repeats direct the VDJ rearrangement. One of the possible mechanisms could be the pairing of two single-stranded protruding ends through these short homologies (Alt and Baltimore, 1982; Roth and Wilson, 1986; Lieber, 1992). This kind of joining reaction occurs frequently during the DNA breakage and rejoining in the nonhomologous recombination in mammalian cells (reviewed by Roth and Wilson, 1986). The existence of P nucleotides suggests that single-stranded broken ends are intermediates during V–(D)–J recombination (Lafaille et al., 1989; Schuler et al., 1991; Kienker et al., 1991). Although there is no direct experimental evidence for single-stranded pairing of the broken ends during V–(D)–J recombination, this would provide an explanation for the resultant junctional sequences generated by short repeats–directed Vγ3–Jγ1 rearrangement.

The presence of sequence-specific or nonspecific DNA binding proteins at the recombination joints may also help to align the broken joints and promote the pairing of short repeats. Although several proteins that bind to the recombinant signal sequences have been identified in the past several years, it remains to be proved that these proteins are required for the V–(D)–J recombination (Lewis, 1994). The recent finding of the Ku80 complex, which binds to free double-stranded DNA ends gives some support to this idea (Taccioli et al., 1994). However, it seems that Ku80 has no sequence preference when it binds the broken DNA ends (Taccioli et al., 1994).

In this study, we also observed several types of predominant Vγ3/Jγ1 junctions that could not be attributed to the presence of germline short repeats or repeats formed by the P nucleotides added precisely at the junctional breakpoint (Figure 2A, types V and VI; Figure 3A, types IV, V, VI, and VII; Figure 4, type X). However, if P nucleotides can also be added at coding ends where 1 or 2 nt have been removed from the double-stranded DNA cleavage site, AT short repeats would be generated that could account for the high frequency of these rearrangements. The possibility of these recessed P nucleotides has been previously proposed (M. Lieber, personal communication). Short repeats generated by addition of the recessed P nucleotides could account for the predominant appearance of several types of Vγ2 junctions (Lafaille et al., 1989). However, there is no direct evidence so far to support recessed P nucleotide addition.

Expression of TdT activity is tightly regulated during embryonic thymic development. The absence of N nucleotides in fetal Vγ3/Jγ1 rearrangement junctions suggested that TdT is not expressed at high level at the time when the rearrangement occurs. In the fetal thymi of TdT transgenic mice, we observed a significant increase in N nucleotide addition and decrease in the frequency of canonical junctions (Figure 5B; Table 1). These results support the idea that lack of TdT activity in the precursors of fetal $V\gamma3^+$ and $V\gamma4^+$ T cells promotes the generation of the invariant repertories in these cells. Thus, regulation of TdT expression during fetal thymic development may play a major role in the programmed development of T cells with invariant repertoires.

Our results clearly demonstrate that increased TdT activity has a inhibitory effect on short repeat–directed rearrangement in vivo. However, the canonical Vγ3–Jγ1 junction is still the predominant junction in TdT transgenic fetal thymus, consisting of 50% of the fetal Vγ3–Jγ1 rearrangement (Figure 5B). Although this may be due to late onset or level of expression from the CD2 promoter, it is also possible that the predominance of the canonical junctions reflects the strong effects of the short homology repeats in inhibiting the action of TdT, perhaps by preventing its binding to ends of the rearranging genes. In adult thymus, 40% of the total in-frame Vγ3–Jγ1 junctions are still the canonical junctions (Komori et al., 1993). This suggests that even when TdT activity is high, such as in the adult thymus, predominant junctions might still be generated by the directing force of the short homology repeats and these junctions may be resistant to N nucleotide addition.

From the above discussion and previous suggestions (Lewis, 1994), it seems that short repeats–directed rearrangement and N nucleotide addition represent two independent processes in V–(D)–J recombination, which have opposite effects on junctional diversity. Short repeats direct the rearrangement to form a restricted repertoire, while TdT increases the repertoire diversity. These two processes compete for the available coding joints during the rearrangement. The final outcome of this competition will be determined by the strength with which the short repeats direct specific rearrangement and the level of TdT activity in the cell where recombination is occurring, and will decide the ultimate repertoire diversity in this system.

Taken together, these data support the idea that short homology repeats have a general function in directing rearrangements during V–(D)–J recombination. Although the presence of short repeats may not change the overall efficiency of recombination (Gerstein and Lieber, 1993), they certainly can determine the outcome of the rearrangement. In fact, certain types of short repeats may be

evolutionarily selected to ensure the generation of specific antibodies against bacterial infection (Feeney, 1991a). Cellular selection may also play a role in further enhancing the junctional homogeneity in certain types of immunoglobulin gene rearrangement; however, it is not required for shaping the TCR repertoires in $V\gamma3^+$ and, presumably, $V\gamma4^+$ T cells.

Although our results clearly indicate that TCR-mediated cellular selection is not required for the generation of γδ canonical sequences, it is still possible that TCR signaling might be needed for the maturation of fetal $V\gamma3^+$ thymocytes. It has been recently demonstrated that in the fetal thymus, $V\gamma3^{lo}/HSA^{hi}$ cells give rise to $V\gamma3^{hi}/HSA^{lo}$ cells and that cyclosporin A (CsA) blocks the appearance of the $V\gamma3^{hi}/HSA^{lo}$ T cells (Leclercq et al., 1993). This suggests a requirement for CsA-sensitive signals, possibly that of the TCR, for maturation of $V\gamma3^+$ thymocytes. Thus, it seems that although TCR-mediated signals are not required for shaping the γδ T cell repertoires, TCR signaling may be needed for the thymic maturation of these T cells (Allison, 1993). It could be postulated that these signals might be used to survey the expression of TCRs on the cell surface and promote the survival of T cells that have in-frame rearrangement.

It remains to be elucidated how short homology repeats can direct the rearrangement and whether single-stranded DNA pairing is involved. The understanding of the functions and mechanisms of the short repeats–directed rearrangement will provide valuable insights towards the understanding of the complicated mechanisms of V–(D)–J recombination.

Experimental Procedures

Construction of Transgenic Mice Carrying γδ Rearrangement Substrates

Plasmid pBSII Vγ2Vγ4Vγ3Jγ1Cγ1 contains Vγ2, Vγ4, Vγ3, Jγ1, and Cγ1 segments and the Cγ1 enhancer in germline configuration (Asarnow et al., 1993). Frameshift mutations were introduced into the Vγ genes to prevent the expression of proteins (Asarnow et al., 1993). Plasmid pBSIIVγ4Vγ3 contains a 4.5 kb Vγ4 and Vγ3 gene fragment cloned in the pBluescript SK(+) vector. Single-stranded DNA was prepared from pBSIIVγ4Vγ3 and used in the site-directed mutagenesis. In vitro mutagenesis was performed using the Bio-Rad Muta-Gene T7 in vitro mutagene kit following the instructions of the manufacturer. Each mutation was confirmed by sequence analysis. Mutated gene segments were cleaved from the pBluescript SKII(+) vector by digesting with NotI and SalI. The resultant 4.5 kb fragment was cloned into the vector pBSII Jγ1Cγ1, which was prepared by digesting plasmid pBSII Vγ2Vγ4Vγ3Jγ1Cγ1 with SalI and NotI to remove the Vγ2Vγ4Vγ3 portion of the plasmid. The resultant plasmid, pBSIIVγ4Vγ3Jγ1Cγ1, was digested with BssHII to isolate a fragment that contained the entire Vγ4Vγ3Jγ1Cγ1 segment. This 22 kb fragment was purified and injected into (C57BL/6 × CBA/J)F2 fertilized eggs. Female mice transplanted with injected embryos were sacrificed at day 17 of the pregnancy. Liver DNA from each fetus was isolated and fetuses carrying the transgene were identified by Southern blot analysis.

Construction of TdT Transgenic Mice

Plasmid HMTdT′ contains the entire open reading frame of a chimeric mouse/human TdT cDNA (Landau et al., 1987). HMTdT′ was partially digested with EcoRI and a 1.5 kb fragment that contains the entire HMTdT open reading frame was isolated. This fragment was cloned into the EcoRI site of ptgar, a modified version of the CD2 minigene expression construct (provided by Dr. A. Winoto). TdT enzyme activity was measured according to Koiwai et al. (1986). Polyclonal anti-TdT antibody was purchased from Life Sciences, Incorporated.

A 7.5 kb SalI–NotI-digested fragment that contains the entire CD2–HMTdT expression cassette was purified and injected into the (C57BL/6 × CBA/J)F2 fertilized eggs. Two male founders were generated and mated with C57BL/6 females to establish the transgenic lines. For timed pregnancies, female mice were examined daily for vaginal plug, which was considered as day 0. Fetuses were harvested at day 17 and Southern blot analyses of liver genomic DNA were performed to identify TdT transgenic fetuses.

PCR Amplification and DNA Sequencing

Fetal thymus was solublized after overnight incubation at 55°C in mouse tail buffer (50 mM Tris [pH 8], 100 mM EDTA, 0.5% SDS, and 400 μg/ml proteinase K). DNA was purified by phenol/chloroform plus chloroform/isoamylalcohol extraction, followed by ethanol precipitation. PCR amplification was performed using the Gene-Amp PCR amplification kit (Perkin Elmer Cetus). Each PCR reaction contained 0.1 μg fetal thymus DNA. Two sets of primers were used for PCR amplification to distinguish transgenic and endogenous rearrangements: Transgenic Vγ3, GAT GAG AAG GAT GAT GCG GAA; Vγ4, GAT GCA TAC ATA CAC TGC CTC. Endogenous Vγ3, GAT GAG AAG GAT GAT GGT ACC; Vγ4, GAT GCA TAC ATA CAC TGG TAC. The 3′ Jγ1 primer was GCG AAG CTT CAG AGG GAA TTA CTA TGA. The PCR products were gel purified and subcloned into pBluescript II SK(+) T vector (Marchuk et al., 1991). DNA sequence analysis was performed using Sanger's dideoxy sequencing methods (Sanger et al., 1977) and Sequenase purchased from US Biochemical.

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